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Creating hybrid proteins by insertion of exogenous peptides into permissive sites of a class A β-lactamase

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Insertion of heterologous peptide sequences into a protein carrier may impose structural constraints that could help the peptide to adopt a proper fold. This concept could be the starting point for the development of a new generation of safe subunit vaccines based on the expression of poorly immunogenic epitopes. In the present study, we characterized the tolerance of the TEM-1 class A β-lactamase to the insertion of two different peptides, the V3 loop of the gp120 protein of HIV, and the thermostable STa enterotoxin produced by enterotoxic *Escherichia coli*. Insertion of the V3 loop of the HIV gp120 protein into the TEM-1 scaffold yielded insoluble and poorly produced proteins. By contrast, four hybrid β-lactamases carrying the STa peptide were efficiently produced and purified. Immunization of BALB/c mice with these hybrid proteins produced high levels of TEM-1-specific antibodies, together with significant levels of neutralizing antibodies against STa.

Gene fusion is a common technique in protein engineering for generating artificial bifunctional proteins for a broad range of applications. Fusion proteins are utilized in protein science research for applications as diverse as immunodetection, protein therapies, vaccine development, functional genomics, analysis of protein trafficking, and analyses of protein–protein or protein–nucleic acid interactions [1]. For example, the use of affinity tags enables different proteins to be purified using a common method as opposed to the highly customized procedures used in conventional chromatographic purification [2]. Most currently used hybrid proteins were created by fusing native or artificial peptides in an end-to-end configuration. However, the three-dimensional structures of many naturally occurring proteins reveal that they are composed of separate domains arising from the insertion of a new stretch of coding sequence at an internal site of an ancestral gene. Engineering such multidomain proteins from internal fusions is more problematic and less frequently described in the literature. There is currently no rule to predict permissive sites within a protein sequence that can be used for the insertion of exogenous polypeptides without altering its intrinsic properties.

Abbreviations
ETEC, enterotoxic *Escherichia coli*; MIC, minimum inhibitory concentration; PSM, pentapeptide scanning mutagenesis.

However, insertion of structural elements inside the host protein can be more advantageous than end-to-end fusions. Backstrom *et al.* showed that internal fusion proteins present a higher resistance to proteolysis than their N-terminal or C-terminal tandem fusion counterparts [3]. The internal insertion of a marker peptide or a protein into strategically important sites of membrane proteins allows analysis of the structural organization of the protein in conditions more similar to the native ones than the utilization of truncated proteins [4]. Betton and co-workers have created bifunctional proteins by insertion of a β-lactamase into the maltodextrin-binding protein. In these hybrid proteins, the activities of both entities were indistinguishable from those of the wild-type proteins [5]. Furthermore, the introduction of a protein loop into internal sites of a protein carrier may impose structural constraints that could help the inserted loop to adopt a fold similar to that observed in the original protein. Such insertion engineering experiments are useful to establish the intrinsic properties of a loop or to characterize its interactions with potential partners. The insertion of epitopes into a carrier protein could also be the starting point for the development of a new generation of safe subunit vaccines [6].

In the present work, the TEM-1 class A β-lactamase was selected as a carrier protein. The three-dimensional structure of TEM-1 is well characterized [7–9]. Like all class A β-lactamases, TEM-1 folds into a structure formed by an α/β-domain and an all-α-domain (Fig. 1A). At the junction between the two domains, a groove harboring the active site is partially covered by an omega loop that is essential for β-lactamase activity. This protein presents several advantages from a practical point of view: it is overexpressed, it can be easily followed during purification, and the permissivity of a large number of insertion sites has already been studied [10]. Furthermore, immunization against β-lactamases may contribute to the struggle against bacterial resistance. Therefore, in this study, we first characterized the tolerance of TEM-1 to the insertion of two different peptides: (a) the V3 loop of the gp120 protein of HIV; and (b) the thermostable STa enterotoxin produced by enterotoxic *Escherichia coli*. The nucleotide and amino acid sequences of these inserts are shown in Fig. 1B. In the second part, we analyzed the use of β-lactamase as a carrier protein in subunit vaccines.

The variable V3 loop is the primary neutralizing determinant of HIV-1. It contains CD4 (Arg315–Ile327) and CD8 (Arg318–Ile327) T-cell epitopes that partially cover a linear B-cell epitope (Ile316–Val325) [11–13]. The presence of these elements renders the V3 loop an interesting target for vaccine development. The 19-mer V3 peptide (Ile314–Gly328) used in this study includes these three epitopes. The second peptide corresponds to the mature form of the heat-stable STa enterotoxin of an enterotoxic *E. coli* (ETEC) strain that can infect cattle. ETEC strains are responsible for significant economic losses in farming, due to the death of newborn calves. The three-dimensional structure of STa has been established by NMR methods [14]. It contains three tightly packed β-strands stabilized by three disulfide bonds that are essential to the toxicity of the peptide [15]. When bound to the guanylin receptor of epithelial cells of the calf intestine, the toxin causes fluid accumulation as a consequence of the activation of guanylate cyclase C and the subsequent accumulation of cGMP in the cells [16]. STa itself is poorly immunogenic, which has hampered the development of efficient vaccines against ETEC thus far.

Results

Insertion of the V3, V3P and STa epitopes at different positions of TEM-1

The tolerance of TEM-1 to short peptide insertions has been examined by pentapeptide scanning mutagenesis (PSM) [10]. The method is based on the random insertion of a variable five amino acid cassette at different positions of a protein. In order to assess how the previously identified insertion site could be influenced by the insertion of large polypeptides, we introduced V3, V3P (36-mer fusion between a β-galactosidase peptide and the V3 sequence arising from *Kpn*I misdigestion) and STa coding sequences within eight different positions of TEM-1. Previously, these positions have been characterized as permissive (two positions), semipermissive (three positions) and non-permissive (one position) by Hallet *et al.*, using the PSM method [10].

Ampicillin resistance conferred by the resulting 18 hybrid proteins was determined and compared to that conferred by the parental proteins (TEMxxx–H) containing the pentapeptide insertion (Table 1). In general, the introduction of the V3 and V3P loop peptides induced a strong decrease in the minimum inhibitory concentrations (MICs). A similar reduction in MICs was found when STa was inserted at positions 198 and 218 of TEM-1. By contrast, insertion of STa at positions 195 and 232 did not change the MIC, and, unexpectedly, STa insertion at positions 216 and 260 even increased resistance to ampicillin. Localization of the β-lactamase by western blot showed that most of the pentapeptide scanning mutants were secreted in a soluble form into the periplasmic space of the bacteria (Table 1). The hybrid proteins TEM37–STa, TEM195–STa,

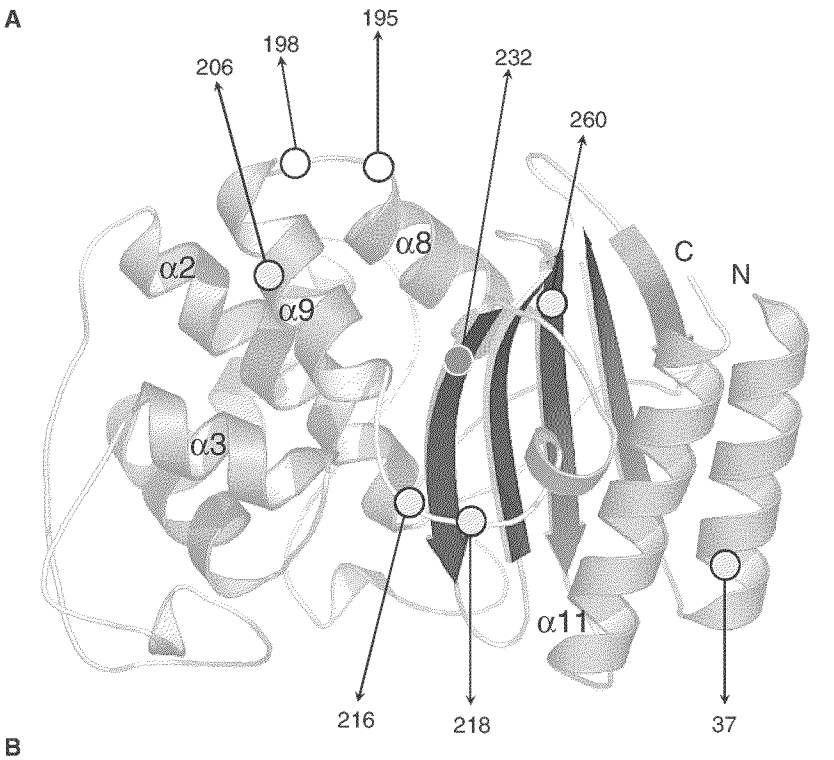

Fig. 1. (A) Three-dimensional structure of TEM-1. Numbers correspond to insertion positions using the ABL consensus numbering system [30]. Positions where inserts have been introduced are indicated by arrows. The permissivity of the sites determined by pentapeptide scanning mutagenesis is color coded: white for highly permissive sites, gray for intermediately permissive sites, and black for nonpermissive sites. Letters C and N indicate, respectively, the C-terminal and N-terminal extremities. (B) Nucleotide and amino acid sequences of the V3 (a), V3P (b) and STa (c) inserts. The V3 peptide sequences corresponded to those of the MN and IIIB HIV-1 isolates [12]. Positively charged amino acids are presented in italics, negatively charged residues are underlined, and disulfide bond-forming cysteines are in bold. Restriction sites are underlined. *Kpn*I and *Sph*I sites are represented, respectively, by bold and italic.

TEM198–V3P, TEM206–STa, TEM216–STa, TEM216–V3P, TEM218–V3P, TEM232–STa and TEM260–STa were at least partially exported to the periplasmic space. TEM195–V3, TEM195–V3P, TEM216–V3 and TEM232–V3P were found in the cytoplasm and/or in the insoluble fraction, where they may form inclusion bodies or be sequestered in the membranes. No production of TEM260–V3P was detected. These results allowed a classification of the different insertion positions. This indicates that positions 195 and 216 tolerate insertions of large peptide sequences, allowing the production of soluble and active hybrid enzymes. Nevertheless, even for these permissive sites, the production of TEM–V3 hybrid proteins and TEM–V3P hybrid proteins was much lower than that of TEM–STa hybrid proteins, and the production of TEM–V3 hybrid proteins was itself much lower than that of TEM–V3P hybrid proteins. It can be concluded that: (a) the structural disturbance caused by the insertion of the V3 or V3P peptide into the β-lactamase scaffold is more important than that caused by STa; and (b) that the 36-mer fusion of the β-galactosidase peptide to the V3 sequence is obviously important for the tolerance of TEM-1 to this V3 peptide sequence. In contrast, insertions in position 232 yielded a soluble protein that was devoid of β-lactamase activity against ampicillin (MIC < 2 $\mu g \cdot mL^{-1}$). The behavior of the variants with insertions in position 260 was totally

Table 1. MIC values of ampicillin for *E. coli* DH5α strains transformed with the different pFH plasmids coding for the different hybrid proteins and western blot analysis. TEM–H, TEM-1 with five amino acid random insertions obtained by the PSM method; TEM–V3, TEM-1 with the V3 epitope-carrying peptides as exogenous insertions; TEM–V3P, TEM-1 with the V3P peptide as exogenous insertions; TEM–STa, TEM-1 with the STa enterotoxin as exogenous insertions. Western blot analyses were performed using proteins isolated from the periplasm (P), cytoplasm (C) and insoluble material (M). Reactivity is shown on a scale of ++ (maximum positive), + (positive) to – (no immunoreaction detected), and ± indicates borderline positive. ND, not determined.

	TEM–H				TEM–V3				TEM–V3P				TEM–STa			
Positions[a]	MIC ($\mu g \cdot mL^{-1}$)	P	C	M	MIC ($\mu g \cdot mL^{-1}$)	P	C	M	MIC ($\mu g \cdot mL^{-1}$)	P	C	M	MIC ($\mu g \cdot mL^{-1}$)	P	C	M
37	50	++	++	±	ND	ND	ND	ND	8	ND	ND	ND	4	+	–	±
195	1024[b]	++	++	±	< 2	–	–	±	64	–	+	++	1024	++	±	+
198	2048[b]	++	±	±	ND	ND	ND	ND	64	+	±	±	256	ND	ND	ND
206	8	–	–	±	ND	ND	ND	ND	< 2	ND	ND	ND	4	±	±	+
216	128	++	+	±	128	–	±	±	8	±	±	±	512	±	–	–
218	128	+	–	+	ND	ND	ND	ND	< 2	+	+	+	16	ND	ND	ND
232	< 2	+	±	–	ND	ND	ND	ND	< 2	–	±	–	< 2	++	+	+
260	16	±	±	±	ND	ND	ND	ND	64	–	–	–	2048	±	–	–

[a] Insertion sites within the TEM-1 scaffold are numbered as in Fig. 1. [b] MIC values obtained with the pFH plasmid coding for TEM195–H and TEM198–H are in agreement with published values for the wild-type [28].

unexpected. The insertion of the pentapeptide into a poorly solvent-exposed area of the protein resulted in an important increase in the MIC as compared to that of the strain producing the native TEM-1 [10]. The insertion of STa in that site restored the production of an active protein and increased the resistance of *E. coli* to ampicillin (MIC = 2048 $\mu g \cdot mL^{-1}$). These differences in production might arise for various and unspecified reasons related to the kinetics of the folding or of the aggregation, to the proteolytic stability, or to the ability of the hybrid protein to be exported to the periplasm.

Production and purification of the hybrid proteins

On the basis of the above results, TEM195–H, TEM195–STa, TEM198–V3P, TEM216–STa, TEM216–V3P, TEM232–STa and TEM260–STa were produced and purified to homogeneity in three chromatographic steps (see Experimental procedures). Stable hybrid protein solutions were obtained after purification for all of the TEM–STa hybrid proteins. In contrast, the TEM–V3P hybrid proteins were degraded after these purification steps. The degree of purity of the different TEM–STa hybrid proteins was higher than 95%, and the yields ranged between 0.4 mg (TEM232–STa) and 3 mg (TEM260–STa) of β-lactamase per liter of culture. The apparent molecular masses of the different hybrid proteins as determined by SDS/PAGE were higher (~ 30 000 Da) than that of TEM-1 (~ 28 000 Da) with the exception of TEM260–STa (~ 28 000 Da) (data not shown). The N-terminal sequence of TEM260–STa was that of the wild-type TEM-1 (HPETL), suggesting that the protein was truncated at the C-terminus. The determination of the molecular mass of TEM260–STa by MS confirmed the loss of the 24 C-terminal residues of TEM-1, corresponding to helix α11. Indeed, the hybrid protein was found to exhibit a molecular mass of 28 905.71 ± 0.56 Da, as compared to the expected molecular mass of 31 601.14 Da. The molecular mass of TEM260–STa minus the C-terminal 24 residues would be 28 907.12 Da.

Enzymatic activity of the hybrid β-lactamases

The steady-state kinetic parameters (k_{cat} and K_m) for hydrolysis of cephaloridine were determined for the different hybrid proteins and compared to those of TEM-1 (Table 2). The insertion of STa at position 195 induced a sixfold decrease in k_{cat} and a fourfold decrease in K_m, so that the catalytic efficiencies of the hybrid and parental enzymes were similar. This indicates that the active site was not significantly altered by the insertion of the enterotoxin at position 195. The catalytic activity of the other hybrid proteins was decreased by a factor larger than 10, due to a large increase in K_m (positions 232 and 260) and a decrease in k_{cat} (position 216).

Enterotoxicity of the TEM–STa hybrid proteins measured by suckling mouse assay

The hybrid proteins (0.05 nmol) exhibited a toxicity that varied with the insertion sites (Fig. 2). Gut/carcass weight ratio values (> 0.085) for STa insertions at

Table 2. Kinetic parameters of the hybrid proteins for cephaloridine.

Proteins	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
TEM-1	1500[a]	670[a]	2.2[a]
TEM195–H	> 1000[b]	> 1000[b]	1 ± 0.1[b]
TEM195–STa	260 ± 20	170 ± 60	1.5 ± 0.4
TEM216–STa	4 ± 1	720 ± 80	0.006 ± 0.001
TEM232–STa	> 340[b]	> 1000[b]	0.34 ± 0.06[b]
TEM260–STa	> 240[b]	> 1000[b]	0.24 ± 0.05[b]

[a] Values for the wild-type TEM-1 are as reported by Raquet *et al.* [29]. [b] Determined by using first-order time courses at [S] << K_m. The time course remained first order up to the concentration given in the K_m column.

positions 195 and 216 were above the toxicity threshold (0.085), indicating that STa retained its biological activity in these insertion sites. In contrast, insertion at positions 232 and 260 produced a toxin of decreased activity, with a gut/carcass weight value < 0.085.

Production of antibodies against the carrier protein TEM-1 and the STa enterotoxin

Purified TEM–STa hybrid proteins were used to immunize BALB/c mice using the protocol described in Table 3, and the production of specific IgG directed against TEM-1 and STa was measured after each injection (Fig. 3). For all the tested hybrid proteins, a positive anti-β-lactamase IgG (anti-TEM) response was observed 2 weeks after the second protein injection (Fig. 3A). In the case of TEM195–H, TEM195–STa and TEM216–STa, the antibody response reached the upper detection limit of the ELISA after two injections. The low variability observed for the humoral responses indicated that the five mice of these groups had a similar response to the injected hybrid β-lactamase. For TEM232–STa and TEM260–STa, the induction of antibodies was more variable inside the group and still increased after the fourth injection. The level of anti-STa IgG was much lower than that of the antibody directed against the carrier protein. Nevertheless, we noted that the humoral response increased with the number of injections and varied according to the position of the STa peptide in the TEM-1 scaffold (Fig. 3B). The highest antibody levels were found in mice vaccinated with TEM195–STa, TEM216–STa and TEM232–STa. Insertion of STa in position 260 induced only a weak antibody response. Humoral responses showed some degree of individual variation, and in each group of five mice, some failed to show detectable antibodies. In the case of TEM260–STa and TEM232–STa, only three of the five treated mice gave a positive response against the enterotoxin.

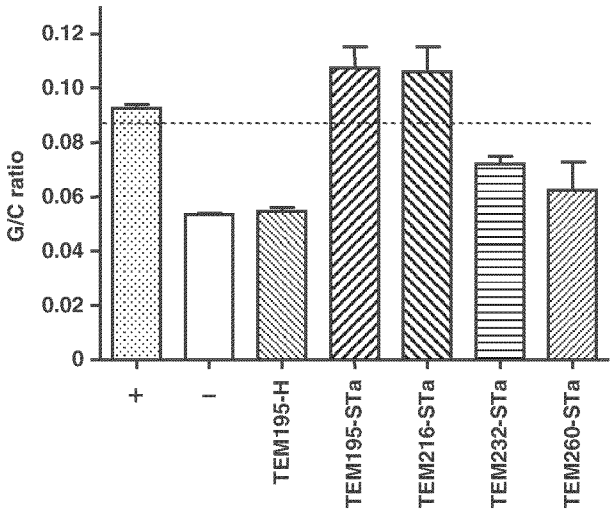

Fig. 2. Enterotoxicity of the hybrid proteins measured by suckling mouse assays. The suckling mouse assay was performed as described by Giannella [31]. The gut/carcass ratios (G/C ratio) are shown for each hybrid protein. The dotted line represents the toxicity threshold (G/C > 0.085) above which the protein samples are considered to be positive (enterotoxic). Positive (+) and negative (–) controls are supernatants of overnight cultures of *E. coli* strains B44 and HS respectively.

Table 3. Immunization time schedule. Days of immunization, bleeding and antibody measurement are identified by a cross (X).

	Days							
	0	14	21	35	42	56	113	127
Immunization	X		X		X		X	
Bleeding	X	X		X		X		X
IgG measurement		X		X		X		X

Neutralization of the STa enterotoxicity

For each group of mice, sera that scored positive in STa-specific ELISA were pooled, and the content of STa-neutralizing antibody was determined by mixing with native STa. Four-fold to 64-fold dilutions of the sera from mice injected either with TEM195–STa or with TEM216–STa were prepared. After incubation, the enterotoxicity of the mixture was determined by suckling mouse assays (Fig. 4). Only pooled sera of TEM195–STa exhibited toxin-neutralizing activity against native STa. This serum pool neutralized the enterotoxicity of native STa at 1 : 4 and 1 : 8 dilutions. The other dilutions (1 : 16 to 1 : 64) resulted in gut/carcass weight values higher than the cut-off (0.085). None of the TEM216–STa serum pool dilutions scored below the cut-off value, indicating the absence of significant amounts of neutralizing STa antibody.

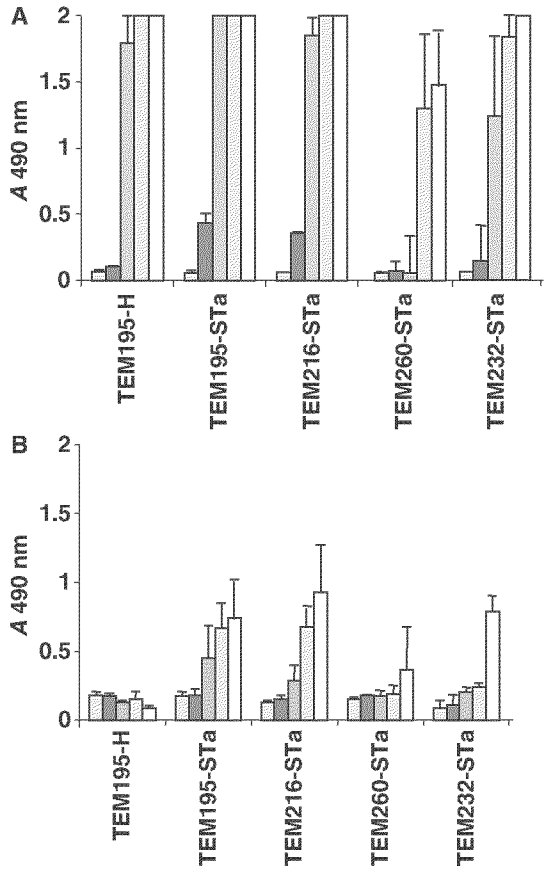

Fig. 3. Antibody production against the carrier TEM-1 (A) and the STa enterotoxin (B). BALB/c mice were immunized with TEM195–H, TEM195–STa, TEM216–STa, TEM260–STa and TEM232–Sta. Each group consisted of five animals. Sera from mice were collected individually on days 0, 14, 35, 56 and 127. IgG antibody response was studied at a serum dilution of 1 : 100.

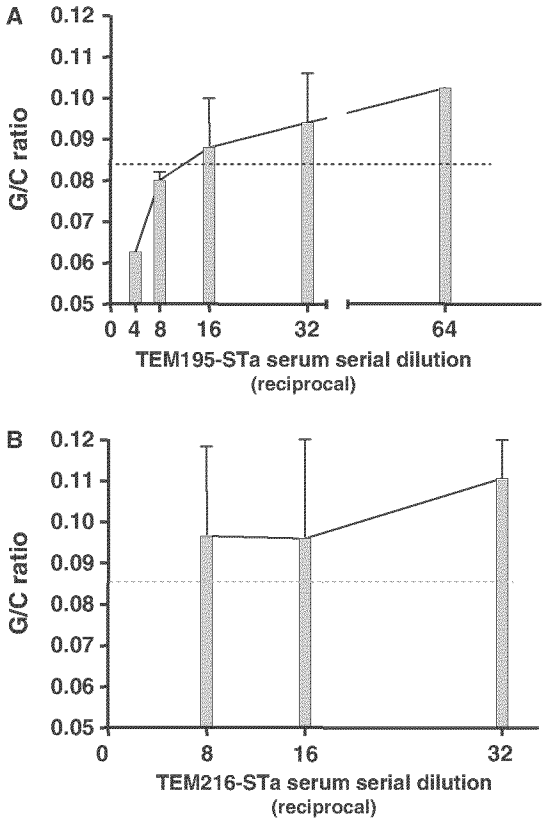

Fig. 4. Neutralization assay of native STa enterotoxin by sera from animals immunized with TEM195–STa (A) and TEM216–STa (B). In each group, sera from mice that showed positive antibody titers were pooled. These samples were diluted in an STa solution (160 ng·mL^{-1}). After a 16 h incubation at 4 °C, the suckling mouse assay was performed as described by Giannella [31]. Gut/carcass weight (G/C) ratios > 0.085 are considered to be positive for STa. The dotted line represents the toxicity threshold above which the samples are considered to be positive (not neutralized).

Discussion

The production of antibodies against a nonimmunogenic peptide is usually achieved by chemically linking the peptide epitope to a carrier protein such as ovalbumin or keyhole limpet hemocyanin [17]. In this work, we investigated the possibility of using TEM-1 as a carrier protein by creating internal fusions, either with the V3 loop peptide of HIV gp120 or with the thermostable STa enterotoxin produced by ETEC.

Previous work by Hallet *et al.* identified permissive, semipermissive and nonpermissive sites for short peptide insertions within TEM-1 [10]. Eight of these positions were selected for inserting sequences corresponding to V3, V3P and STa, respectively.

The insertion site at position 37 (Leu37) is located in helix α1 and is poorly exposed to the solvent. Leu 37 is the only conserved residue of the decapeptide sequence surrounding this position in all known class A β-lactamases. Pentapeptide scanning mutagenesis of TEM-1 showed that position 37 was semipermissive to insertion. TEM37–H was produced in the periplasm but showed reduced activity against ampicillin. Increasing the length of the insert induced a sixfold decrease in the MIC value, despite the fact that the protein was exported to the periplasmic space. The poor protein stability could be related to the presence of a proline in the heterologous sequence. The presence of this residue is not favorable for the formation of stable α-helices. The collapse of helix α1 probably disturbs the β-lactamase fold.

Palzkill *et al.* showed that the loop located between helix α8 and helix α9 (residues 195–200) can be randomly modified without loss of enzymatic activity [18]. This observation was in good agreement with the finding that pentapeptide insertion at position 195 does

not significantly alter the activity and solubility of the protein [10]. Consistent with this, the addition of the 18 residue heat-stable enterotoxin STa in position 195 did not affect the behavior of the TEM β-lactamase. The catalytic efficiencies of TEM195–STa and TEM-1 against ampicillin and cephaloridine were found to be similar. In addition, we also demonstrated that the enterotoxicity of STa in TEM195–STa was maintained. These observations suggest that the folds of the carrier protein and the inserted peptide are very similar to those of their native counterparts. In contrast, the insertion of the V3 and V3P sequences had effects on the stability of TEM-1. Despite the fact that the V3P protein seems to be at least partially exported to the periplamic space, no soluble and stable hybrid protein seemed to be produced. Similar results were obtained for insertions in position 198.

Residue 206 of TEM-1 is located on the solvent-exposed helix α9. Therefore, peptide insertions at this position probably destabilize the helix and thus the complete protein.

The loop connecting helix α9 and helix α10 (residues 213–220) is exposed to solvent and is poorly conserved among the other class A β-lactamases. Insertion at positions 216 or 218 of the loop yielded soluble and secreted hybrid proteins, except for TEM216–V3. TEM216–STa remained active against ampicillin and cephaloridin. However, its catalytic efficiency decreased 300-fold as compared to TEM-1. As noted for the other positions, insertion of STa appeared to be more easily accepted by the β-lactamase than insertion of V3 and V3P.

Insertion at position 232 occurs in the hydrophobic core of the protein located near the KT/SG motif, which is conserved in all class A β-lactamases. The hybrid protein was still active against cephaloridine. Nevertheless, the low MIC value for ampicillin indicated that the production and/or enzymatic activity of the protein were affected. Although this position was described as poorly tolerant to sequence modifications, a soluble and active enzyme could be produced.

Finally, insertion at position 260 occurs in the N-terminal end of strand β5. This insertion modified the structure of the protein so that its susceptibility to proteolysis was increased. In fact, a protein with the last α-helix (α11) deleted was obtained. Nevertheless, TEM260–STa could efficiently hydrolyze cephaloridine. Its catalytic efficiency was only decreased 10-fold as compared to the wild-type β-lactamase.

Immunization of BALB/c mice with the various hybrid proteins allowed the production of TEM-1-specific IgG antibodies, although insertion of STa at positions 260 and 232 did not induce a strong TEM-1-specific antibody response before the third injection. These data can be explained by the fact that the C-terminal helix of TEM-1 contains an immunodominant B-cell epitope. Insertion of STa at positions 232 and 260 affects the hydrophobic core of β-lactamase, and may therefore disturb the overall fold of the protein. As a consequence, the accessibility of this immunodominant epitope could be altered. Moreover, the insertion of STa at position 260 yielded a protein that was more sensitive to proteases, leading to deletion of helix α11.

Interestingly, immunization with TEM–STa hybrid proteins yielded a low-titer humoral response against the normally nonimmunogenic enterotoxin. However, the immune response against the carrier is clearly higher than that against the enterotoxin. This shows that the carrier B-cell epitopes are immunodominant. As already observed for TEM-1, the immune response against STa at positions 260 and 232 was lower than the response against STa at positions 195 and 216. The STa neutralization experiments performed in suckling mouse assays showed the presence of neutralizing antibodies in sera from mice vaccinated with TEM195–STa but not with TEM260–STa, indicating that the position of the insertions in TEM-1 is critical for the induction of neutralizing antibodies. The results obtained here for recombinant proteins are in good agreement with results previously obtained by DNA vaccination [19]. In both cases, the best antigen was TEM195–STa. The transient expression of this hybrid protein obtained by DNA vaccination or its injection into mice yielded the highest immune response against TEM-1 (data not shown). In order to favor a better immune response to STa, we will investigate other permissive insertion positions in TEM-1. In addition, substitution of the cysteine of STa could lead to a better antigen, as already suggested by DNA vaccination [19]. In addition, the high TEM-1 immunogenicity indicates that TEM-1 contains functional T-helper epitopes. The T-helper epitopes are needed to induce an immune response against a hapten. However, the immune response against the carrier is clearly higher than that against the enterotoxin. In order to reduce the immunodominance of the carrier B-cell epitopes, we will identify and generate mutations by site-directed mutagenesis.

In this study, we used TEM-1 as a carrier to induce neutralizing antibodies against the nonimmunogenic STa enterotoxin from ETEC. Hybrid proteins were created by insertion of this STa peptide in different positions within the enzyme scaffold. Immunization of BALB/c mice with one of these hybrid proteins induced low levels of neutralizing antibodies against STa. Moreover, we also created bifunctional

proteins in which the activities of both entities were conserved.

Experimental procedures

Antibiotics, chemicals and enzymes

Nitrocefin was purchased from Unipath Oxoid (Basingstoke, UK). Benzylpenicillin and tetracycline were purchased from Sigma (St Louis, MO, USA), 5-bromo-4-chloro-3-indoyl-phosphate and 4-nitroblue tetrazolium chloride from Boerhinger (Mannheim, Germany), and isopropyl-thio-β-D-galactoside from Eurogentec (Liège, Belgium). Restriction enzymes were purchased from Gibco BRL Life Technology (Merelbeke, Belgium), Boehringer (Mannheim, Germany) and Eurogentec (Liège, Belgium), T4 ligase and calf intestine alkaline phosphatase from Boerhinger (Mannheim, Germany), *Pfu* DNA polymerase from Promega Corp. (Madison, WI, USA) and *Vent* DNA polymerase from New England BioLabs Inc. (Beverly, MA, USA).

Plasmids, bacterial strains and culture conditions

Plasmids pFH37, pFH195, pFH198, pFH206, pFH216, pFH218, pFH232 and pFH260 are pBR322 derivatives coding for the TEM-1 mutants, and were obtained by random insertion of variable pentapeptides into the coding sequence of the β-lactamase gene (*bla*) according to the PSM method [10]. Numbers in the plasmid names refer to the positions of the pentapeptide insertions in the mature TEM-1 amino acid sequence. The corresponding mutant proteins are designated as TEMxxx–H, where xxx refers to the plasmid number. Each plasmid carries a unique *Kpn*I restriction site that was introduced together with the pentapeptide insertion [10]. *E. coli* strain DH5α was used for the plasmid propagation and cloning experiments. Production of the different proteins was performed in the *E. coli* JM109 strain. Plasmids were purified with the Nucleobond PC 100 kit (Macherey-Nagel, Düren, Germany). DNA fragments were separated in a 1% agarose gel and purified with the GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech Inc., Uppsala, Sweden). All DNA restriction, ligation and dephosphorylation experiments were carried out following the supplier's recommendations or the protocol described by Sambrook *et al.* [20].

Construction of synthetic DNA linkers coding for the V3 and STa epitopes

Double-stranded DNA linkers coding for the V3 epitope and the STa peptide were constructed by annealing pairs of synthetic oligonucleotides of the corresponding sequences (Fig. 1B). *Kpn*I restriction sites were introduced at both ends of the nucleotide sequences. The oligonucleotides were annealed by successive cycles of forced heating to 90 °C and cooling to room temperature. The products were ligated into the pCR II vector (Invitrogen, Belgium) and transformed in *E. coli* DH5α. The resulting pCR–V3 and pCR–STa plasmids were purified and the nucleotide sequences of the inserts were verified.

Construction of the hybrid β-lactamases

The V3 and STa epitopes were inserted at eight different positions in TEM-1 (positions 37, 195, 198, 206, 216, 218, 232 and 260), using the pentapeptide insertion mutants produced by PSM [10]. Plasmids pFH37, pFH195, pFH198, pFH206, pFH216, pFH218, pFH232 and pFH260 were digested by *Kpn*I and subsequently dephosphorylated by calf intestine alkaline phosphatase. The linearized plasmids were purified from 1% agarose gel by the GFX DNA and Gel Band Purification Kit. Plasmids pCR–V3 and pCR–STa were digested by *Kpn*I. Two fragments coding for the V3 epitope (V3 and V3P) and one for STa were purified on an 8% polyacrylamide gel [20]. The V3P fragment was obtained from partial digestion of the pCR II vector by the *Kpn*I restriction enzyme. V3P is a 36-mer fusion between a β-galactosidase peptide and the V3 sequence. The V3P fragment was inserted in order to assess how the insertion site could be influenced by the insertion of a larger polypeptide than the V3 epitope. The fragments were introduced into the different linearized pFH plasmids to yield pFH37–V3P, pFH37–STa, pFH195–V3, pFH195–V3P, pFH195–STa, pFH198–V3P, pFH198–STa, pFH206–V3P, pFH206–STa, pFH216–V3, pFH216–V3P, pFH216–STa, pFH218–V3P, pFH218–STa, pFH232–V3P, pFH232–STa, pFH260–V3P, and pFH260–Sta, respectively.

Measurement of the MIC

Portions (0.1 mL) of an overnight culture of the different *E. coli* strains transformed with one of the pFH–V3, pFH–V3P or pFH–STa plasmids were added to 10 mL of fresh LB broth supplemented with 12.5 $\mu g \cdot mL^{-1}$ tetracycline. The cultures were grown at 37 °C until their absorbance at 600 nm reached 1 absorbance unit. The cultures were then diluted 1000-fold in 5 mL of LB broth containing increasing concentrations of ampicillin (from 2 to 1024 $\mu g \cdot mL^{-1}$) in addition to 12.5 $\mu g \cdot mL^{-1}$ tetracycline. The cultures were incubated for 18 h at 37 °C. The MICs correspond to the lowest ampicillin concentrations that completely inhibited bacterial growth.

Cellular localization of the hybrid β-lactamases

The localization of the different TEM–V3 and TEM–STa hybrid proteins was examined by western blot analysis.

Portions (0.1 mL) of an overnight culture of the different *E. coli* strains transformed with one of the pFH–V3, pFH–V3P or pFH–STa plasmids were added to 10 mL of fresh LB broth supplemented with 12.5 $\mu g \cdot mL^{-1}$ tetracycline. The cultures were incubated at 37 °C until their absorbance at 600 nm reached 0.6 absorbance units. Five milliliters of the culture was centrifuged at 10 000 ***g*** for 4 min at 4 °C. The pellet was suspended in 500 μL of 30 mM Tris/HCl (pH 8) containing 5 mM EDTA and 27% sucrose. Lysozyme (100 $\mu g \cdot mL^{-1}$) was added to the suspension, and the mixture was incubated for 10 min in an ice/water bath. After 10 min, $CaCl_2$ was added to a final concentration of 15 mM. The bacteria were collected by centrifugation at 2500 ***g*** for 10 min. The supernatant corresponds to the periplasmic fraction of the *E. coli* cells. The pellet was suspended in 500 μL of 30 mM Tris/HCl (pH 8) and subjected to three freeze–thaw cycles. The solution was centrifuged at 20 000 ***g*** for 20 min at 4 °C. The soluble fraction corresponds to the cytoplasm, and the insoluble material to membranes and inclusion bodies. The insoluble fraction was suspended in 500 μL of 30 mM Tris/HCl (pH 8). Portions (15 μL) of each fraction (periplasm, cytoplasm, and membranes) were loaded onto a 10% SDS/PAGE gel. Proteins were electrotransferred onto a nitrocellulose membrane (Millipore Corporation, Madison, WI, USA) and incubated with rabbit polyclonal antibodies against TEM. Goat anti-(rabbit IgG) coupled to alkaline phosphatase (Bio-Rad, Hercules, CA, USA) were added (according to the supplier's recommendations). The primary and secondary antibodies were diluted 1000-fold and 3000-fold respectively in NaCl/Tris containing 1% (w/v) BSA and 0.5% (v/v) Tween-20. Positive protein bands were revealed by 5-bromo-4-chloro-3-indoyl-phosphate and 4-nitroblue tetrazolium chloride (Roche Applied Science, Basel, Switzerland), which form a precipitate after the action of alkaline phosphatase.

Production and purification of the TEM–STa hybrid proteins

Preculture of *E. coli* JM109 pFH195, pFH195–STa, pFH216–STa, pFH232–STa and pFH260–STa was performed at 18 °C by inoculation of 400 mL of fresh LB broth with a single colony. After 65 h of growth, the precultures were added to 4 L of LB broth. The cultures were incubated at 18 °C for 18 h. The periplasmic fractions were isolated as described above, and dialyzed overnight against 10 L of 20 mM Tris/HCl (pH 8) (buffer A). The extract was loaded onto a High Load Q Sepharose 36/10 column (Pharmacia, Uppsala, Sweden) equilibrated with buffer A. The different proteins were eluted by a linear NaCl gradient (0–0.5 M) over five column volumes. The fractions containing the hybrid proteins – identified either by their β-lactamase activity or by western blot using polyclonal antibodies against TEM – were pooled and dialyzed against 100 volumes of 20 mM Mes (pH 6.5) (buffer B). The solution was then loaded onto the High Load Q Sepharose 36/10 column equilibrated with buffer B. Elution of the hybrid proteins was performed with the help of a linear salt gradient (0–0.5 M NaCl) over five column volumes. The fractions containing the different hybrid proteins were pooled, concentrated by ultrafiltration (cut-off = 10 000 Da), and filtered through a 0.22 μm filter. The pooled and concentrated fractions were then loaded onto a Superdex 75HR 5/20 column (Pharmacia) to eliminate low molecular mass contaminants. The samples were concentrated by ultrafiltration (cut-off = 10 000 Da) to a final concentration of 2 $mg \cdot mL^{-1}$, and stored at −20 °C in 25 mM sodium phosphate buffer (pH 7). The purity of the different hybrid proteins was estimated by SDS/PAGE.

N-terminal sequencing of protein

N-terminal sequencing was performed by the Edman degradation procedure as described by Han *et al.* [21].

MS

ESI MS of purified proteins was performed in collaboration with E. DePauw's laboratory (Laboratory of Physical Chemistry, University of Liège). The exact masses of the hybrid proteins were determined in positive-ion mode on a Q-Tof Ultima mass spectrometer (Micromass, Newbury, UK) fitted with a nanospray source and using homemade gold-coated borosilicate glass emitters. Before injection into the mass spectrometer, the samples were desalted by performing three cycles of concentration–dilution (fivefold) in 0.1% formic acid/acetonitrile (50 : 50, v/v), using an Ultrafree-MC centrifugal filter device (Millipore) with a 10 000 Da nominal molecular mass limit. Final protein concentrations varied from 2 to 5 μM. Calibration was performed with horse heart myoglobin.

Determination of kinetic parameters

The steady-state kinetic parameters k_{cat} and K_m of TEM195–H, TEM195–STa, TEM216–STa, TEM232–STa and TEM260–STa were measured against cephaloridine ($\Delta\varepsilon_{260} = -10\,000\ M^{-1} \cdot cm^{-1}$), with the help of a Uvikon 860 spectrophotometer linked to a microcomputer via an RSC232 interface. The experiments were performed at 30 °C in 50 mM phosphate buffer (pH 7). The different parameters were obtained as described by De Meester *et al.* [22].

Suckling mouse assay

The toxicity of STa was estimated by suckling mouse assays. This assay measures the fluid secretion into the intestinal lumen of newborn mice after injection of the

sample into their stomach [23]. (The protocol was accepted by the Ethical Committee of the University of Liège, 26 April 2000, protocol 86.) To test the toxicity of the produced hybrid proteins, a group of five newborn mice received 0.5 nmol of the different TEM–STa hybrid proteins. After 3 h at 22 °C, the animals were killed, and gut/carcass weight ratio was measured. A gut/carcass ratio ≥ 0.085 was considered to be positive. The positive and negative controls were the supernatants of overnight broth cultures of *E. coli* strains B44 [24] and HS [25], respectively.

Immunization

Female BALB/c mice were injected four times, at 3 week intervals with 50 μg of one of the different TEM–STa hybrid proteins diluted in NaCl/P_i containing QuilA as adjuvant (Spikeoside, Isotech, Ab, Luleå, Sweden). The experimental schedule and the different experimental groups are indicated in Table 3.

Measurement of specific IgG antibody production

TEM-1-specific and STa-specific antibodies were detected by ELISA in the mouse sera. For the detection of antibodies against the carrier TEM-1, 96-well microtiter plates (Maxisorp; Nunc-Immunoplate, Roskilde, Denmark) were coated overnight at 4 °C with 250 ng per 50 μL of β-lactamase per well. For the detection of antibodies against STa, 96-well microtiter plates were coated overnight at 4 °C with 250 ng per 50 μL of glutathione *S*-transferase–STa per well. The plates were washed three times with NaCl/P_i. Then, 100 μL of blocking buffer (NaCl/P_i containing 3% BSA) was added to each well, and plates were incubated at 37 °C for 60 min. After washing three times with NaCl/P_i containing 0.05% Tween-20, 50 μL of a 100-fold diluted serum in blocking buffer was added to the wells. Plates were incubated for 1 h at 37 °C, and then washed three times with NaCl/P_i containing 0.05% Tween-20. Fifty microliters of horseradish peroxidase-labeled sheep anti-(mouse IgG) (Sigma, St Louis, MO, USA) were added (dilution following manufacturer's instructions). Plates were washed three times with NaCl/P_i containing 0.05% Tween-20. The reaction was developed using Sigma Fast *o*-phenylenediamine dihydrochloride tablets set for 10 min, and stopped by addition of 1 M H_2SO_4. The absorbance of the solution was read at 490 nm (Labsystems Multiskan Multisoft; TechGen International, London, UK).

Antibody neutralization of STa enterotoxicity

The native STa was isolated from a culture of *E. coli* B44 as described previously [26,27]. To test the neutralization activity of the anti-STa sera on the biological activity of native STa, 0.5 nmol of native STa was incubated with various dilutions of the sera raised with the TEM195–STa and TEM216–STa antigens. Four-fold to 64-fold dilutions were performed in 0.7 mL of NaCl/P_i. The different STa toxin–serum mixtures were incubated at 4 °C for 16 h with shaking. The residual toxicity of the samples was tested by the suckling mouse assay as described above.

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